Another Brick in the Wall: the role of the actinobacterial cell wall in antibiotic resistance, phylogeny and development

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CHAPTER 4

The peptidoglycan composition of *Kitasatospora setae* and *Kitasatospora viridifaciens* is highly modified during sporulation

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Supplementary material available at Appendix 1

ABSTRACT:

Members of the genus *Kitasatospora* are Gram-positive actinomycetes that belong to the family of *Streptomycetaceae*, and strongly resemble *Streptomyces* in terms of their genetics and morphology. However, there are also differences, such as that key developmental genes are missing and their cell wall composition differs significantly. *Kitasatosporae* have a unique switch in peptidoglycan composition during their life cycle, producing a vegetative mycelium with *meso*-DAP in the peptidoglycan, while the spore peptidoglycan contains LL-DAP. Here, we investigate the biological implications of the growth phase-dependent changes in the PG composition. Our work shows that the peptidoglycan of *Kitasatospora setae* and *Kitasatospora viridifaciens* changes composition between the point of vegetative growth and sporulation. An *ssgB* null mutant of *K. viridifaciens*, which fails to sporulate (Ramijan *et al.*, 2018), accumulated muropeptides with a modification at the site of MurNAc, a higher amount of hydrolyzed dimers as compared to the parent strain and has minimal amounts of peptidoglycan with LL-DAP. The data suggests that at the switch from vegetative growth to reproductive growth, *Kitasatospora* species actively hydrolyze most of the vegetative mycelium, potentially in a *meso*-DAP directed manner in order to produce reproductive spores.

INTRODUCTION:

Actinomycetes are Gram-positive bacteria well known for their capability to produce a wide array of secondary metabolites, among which most of our clinical antibiotics (Barka *et al*., 2016; Hopwood, 2007). The search for novel antibiotic-producing actinobacteria has resulted in the isolation of an huge diversity of taxonomically described actinomycetes. Many of these belong to the genus *Streptomyces*, of which no fewer than 550 type species have been described (Kämpfer, 2012). Besides *Streptomyces*, the family of *Streptomycetaceae* includes *Kitasatospora* and *Streptacidiphilus* (Goodfellow, 2012; Labeda *et al*., 2012; Labeda *et al*., 2017). *Kitasatospora* are a sister genus to *Streptomyces* with an indistinguishable morphology, which has caused many *Kitasatospora* species to be mis-assigned to the genus *Streptomyces* (Girard *et al*., 2014; Ramijan *et al*., 2017).

During growth of *Kitasatospora*, a single spore germinates and develops vegetative hyphae, which extend by tip growth and branching, thereby developing an intricate vegetative mycelium. Reproductive growth starts with the development of aerial hyphae, which grow into the air and eventually differentiate into long spore chains (Barka *et al*., 2016). Development from vegetative to aerial mycelium is regulated by the *bld* genes, named after the nonsporulating phenotype of mutants, whereby colonies have a 'bald' appearance; spore formation is regulated by the *whi* genes, so-called because *whi* mutants are white, due to their failure to produce the grey-pigmented spores (Chater, 1972; Merrick, 1976). *Kitasatosporae* lack the regulatory genes *bldB* and *whiJ* that are important for development in *Streptomyces*, and the cytoskeletal gene *mbl*, for an MreB-like protein (Celler *et al*., 2013). BldB is a small protein involved in the regulation of development and antibiotic production, WhiJ mediates repression of *Streptomyces* development (Ainsa *et al*., 2010; Pope *et al*., 1998).

These data suggest that at least some aspects of *Kitasatospora* development is regulated different from *Streptomyces* (Girard *et al*., 2014). Another major difference lies in the so-called SsgA-like proteins. Sporulation of actinobacteria requires one or more proteins that belong to the SsgA-like proteins (SALPS) (Traag, 2008; van Dissel, 2014). All actinobacteria that sporulate contain an orthologue of *ssgB*, while more complex actinomycetes like streptomycetes encode several SALPS (Noens *et al*., 2005). Of these, *ssgA* and *ssgB* are required for sporulation in streptomycetes; mutants deleted for *ssgA* only form spores on minimal media (van Wezel *et al*., 2000), while *ssgB* mutants fail to sporulate under any growth condition (Keijser *et al*., 2003). SsgB recruits FtsZ to septum sites so as to initiate sporulation-specific cell division (Willemse *et al*., 2011). Several *Kitasatosporae* lack *ssgA* and/or its regulatory gene *ssgR*, again suggesting that the control of development is different in *Kitasatospora* and *Streptomyces*.

One determining feature that sets *Kitasatospora* apart from *Streptomyces* is found in the cell wall composition: diaminopimelic acid (DAP) is a diagnostic features in actinomycetales, and this modified amino acid contains two stereocenters of which two stereoisomers are found in bacteria, LL-DAP and L,D(*meso*)-DAP (Lechevalier and Lechevalier, 1965). *Streptomyces* PG exclusively contains LL-DAP, but *Kitasatospora* species carry *meso*-DAP in PG of the vegetative mycelium and LL-DAP in spore PG, as shown in Figure 1 (Takahashi, 2017; Zhang *et al*., 1997). Analysis of the cell wall composition of *Streptomyces* failed to identify muropeptides that were unique for either vegetative mycelia or spores; instead, the major differences were seen in

the abundance of muropeptides (van der Aart *et al*., 2018).

The switch from *meso*- to LL-DAP in the PG between vegetative mycelium and spores of *Kitasatospora* species suggests a form of regulation at the point of the cell wall construction (Takahashi, 2017). In this study, we analyzed the peptidoglycan composition of mycelia and spores of *Kitasatospora setae* KM 6054^{T} and *Kitasatospora viridifaciens* DSM40239. Analysis of the muropeptides by MS/MS revealed major changes in cell wall composition between vegetative mycelium and spores of both strains. An *ssgB* deletion mutant of *K. viridifaciens* showed a higher degree of cell wall hydrolysis and an unindentified modification at the site of MurNAc.

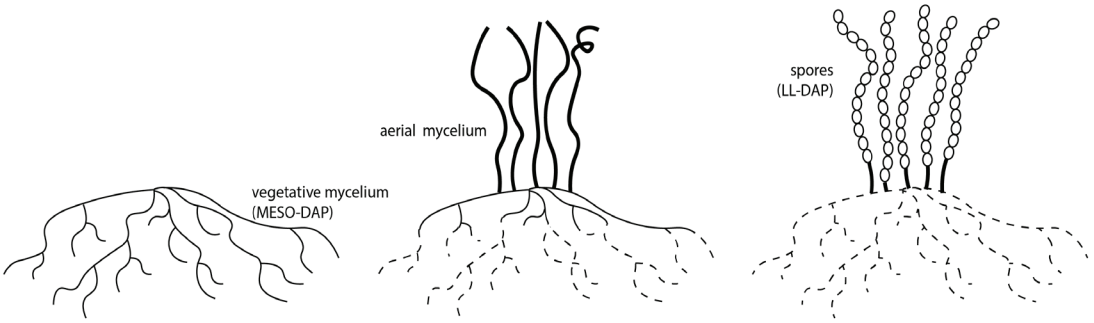

Figure 1. *Kitasatospora* produces a vegetative mycelium with LL-DAP, followed by an aerial mycelium that develops into spores with LL-DAP. Over the course of development, the vegetative mycelium is broken down during a process of programmed cell death, indicated by dashed lines.

MATERIAL AND METHODS

Bacterial strain and culturing conditions:

Kitasatospora setae KM 6054^T and *Kitasatospora viridifaciens* DSM40239 are both obtained from the DSMZ. *K. viridifaciens* was grown on MYM-medium, *K. setae* was grown on ISP-2. Spores are isolated by craping them off with a cotton ball and drawing the solution with a syringe. The *ssgB*-mutant of *K. viridifaciens* was generated by Karina Ramijan (Ramijan *et al*., 2018).

PG extraction

Cells were lyophilized for a biomass measurement, 10 mg biomass was directly used for PG isolation. PG was isolated according to Kühner *et al.* (Kühner *et al*., 2014), using 2 mL screw-cap tubes for the entire isolation. Biomass was first boiled in 0.25% SDS in 0.1 M Tris/HCl pH 6.8, thoroughly washed, sonicated, treated with DNase, RNase and trypsin, inactivation of proteins by boiling and washing with water. Wall teichoic acids were removed with 1 M HCl (van der Aart *et al.*, 2018). PG was digested with mutanolysin and lysozyme (Arbeloa *et al.*, 2004). Muropeptides were reduced with sodium borohydride and the pH was adjusted to 3.5-4.5 with phosphoric acid.

LC-MS analysis of monomers

The LC-MS setup consisted of a Waters Acquity UPLC system (Waters, Milford, MA, USA) and a LTQ Orbitrap XL Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an Ion Max electrospray source.

Chromatographic separation was performed on an Acquity UPLC HSS T3 C_{18} column (1.8 μm, 100 Å, 2.1 × 100 mm). Mobile phase A consist of 99.9% H_2O and 0,1% Formic Acid and mobile phase B consists of 95% Acetonitrile, 4.9% H_2O and 0,1% Formic Acid. All solvents used were of LC-MS grade or better. The flow rate was set to 0.5 ml/min. The binary gradient program consisted of 1 min 98% A, 12 min from 98% A to 85% A, and 2 min from 85% A to 0% A. The column was then flushed for 3 min with 100% B, the gradient was then set to 98% A and the column was equilibrated for 8 min. The column temperature was set to 30°C and the injection volume used was 5 μL. The temperature of the autosampler tray was set to 8°C. Samples were run in triplicates.

MS/MS was done both on the full chromatogram by data dependent MS/MS and on specific peaks by selecting the mass of interest. Data dependent acquisition was performed on the most intense detected peaks, the activation type was Collision Induced Dissociation (CID). Selected MS/MS was performed when the resolution of a data dependent acquisition lacked decisive information. MS/MS experiments in the ion trap were carried out with relative collision energy of 35% and the trapping of product ions were carried out with a q-value of 0.25, and the product ions were analyzed in the ion trap., data was collected in the positive ESI mode with a scan range of *m*/*z* 500–2500 in high range mode. The resolution was set to 15.000 (at m/z 400).

Data analysis

Chromatograms were evaluated using the free software package MZmine (http://mzmine.sourceforge.net/ (Pluskal *et al.*, 2010)) to detect peaks, deconvolute the data and align the peaks. Only peaks corresponding with a mass corresponding

to a muropeptide were saved, other data was discarded. The peak areas were exported and a final table which shows peak areas as percentage of the whole was produced in Microsoft Excel.

Muropeptide identification

The base structure and modifications of *S.coelicolor* PG has been resolved (Hugonnet *et al.*, 2014). Different combinations of modifications were predicted and the masses were calculated using ChemDraw Professional (PerkinElmer). When a major peak had an unexpected mass, MS/MS helped resolve the structure. MS/MS was used to identify differences in cross-linking and to confirm predicted structures.

DAP characterization

2-3 mg of freeze-dried material was dissolved in 200 μL 6M HCl and boiled for 16 hours. After cooling, the tubes were transferred to the rotary evaporator and dried. The remaining material was dissolved in 100 μL H_2O. 2μL of each sample was applied to the baseline of a cellulose TCL-plate. The tank was filled with Methanol:H_2O:Pyridine:12M HCl (32:7:4:1) (Lechevalier *et al.*, 1971).

RESULTS

Diaminopimelic acid composition in vegetative mycelium and spores.

Mycelia from *K. setae* and *K. viridifaciens* were harvested from solid ISP-2 or MYM agar plates at 16 h (vegetative growth), 30 h (aerial growth), 42 h (onset of sporulation) and 64 h (mature spores) and spores separated from the mycelia by filtering through cotton wool. Mycelium of the non-sporulating *ssgB* null mutant of *K. viridifaciens* was harvested after 64 h, when abundant aerial hyphae were formed, as shown in figure S1. This mutant was used to analyse aerial mycelium after prolonged incubation, in the absence of spores. Then, cell wall material was isolated from all the mycelia and spores as described (Lechevalier *et al*., 1971). The diaminopimelic acid (DAP) stereochemistry of the samples was assessed by TLC. In agreement with the literature, during vegetative growth (16 h) only *meso*-DAP was identified, while spore samples exclusively contained LL-DAP. Interestingly, *K. setae* cell walls contained a major amount of LL-DAP in 42 h samples and apparently exclusively LL-DAP was found in 64 h samples. For *K. viridifaciens*, this effect was less pronounced, but mycelia that had grown for 64 h had a DAP profile that is highly similar to that of spores. This suggests that after 64h, a large part of the vegetative mycelium has been used as a substrate for the aerial mycelium. The *ssgB* deletion mutant of *K. viridifaciens* carried mostly *meso*-DAP after 64 h of growth, with trace amounts of LL-DAP, suggesting that aerial hyphae are modified before the point of sporulation (Figure S2).

Peptidoglycan analysis

Cell wall material of *K. setae* and *K. viridifaciens* was isolated from the same samples as the DAP isolation, from both *K. setae* and *K. viridifaciens* after 16 h, 30 h , 42 h, and 64 h of growth. Spores were filtered through cotton wool, mycelium from the ssgB-mutant of K. viridifaciens was harvested at 64 h. PG was isolated as described previously (van der Aart *et al*., 2018). Most muropeptides found in the profiles of *K. setae* and *K. viridifaciens* have previously been described for *S. coelicolor*. The basal structure of the muropeptides is a glycan backbone consisting of GlcNAc and MurNAc, where a pentapeptide chain is attached to MurNac consisting of L-Ala-D-Glu-*meso*/LL-DAP-D-Ala-D-Ala with Gly attached to the DAP residue. Cross-links can be formed via the DAP-linked Gly, which then connects to the opposite D-Ala in the case of a 3-4 cross-link or DAP in the case of a 3-3 cross-link.

Modifications to monomers

After 16 h of growth, the PG from both *Kitasatospora* species contained only *meso*-DAP. From 30 h onwards, their PG contained minor amounts of LL-DAP, which is seen in the muropeptide profile where double peaks started to appear. The major switch from *meso*-DAP to LL-DAP occurred with a switch in PG modification, whereby both species of *Kitasatospora* showed major changes in cell wall composition at the time of sporulation. Modification to the PG structure involve: peptidolytic cleavage of amino acids to generate tetra-, tri-, di- or monopeptides, loss of Gly, amidation of Glu to glutamine (Gln) (Figueiredo *et al*., 2012), and amidation of DAP (Levefaudes *et al*., 2015). Modifications to the glycan strand include de-acetylation of MurNAc to MurN, as often seen in older mycelia and spores of *Streptomyces* and amidation of the glycan strands whereby MurNAc-GlcNAc is cleaved off, to give a dimer with a single set of glycans (van der Aart *et al*., 2018) (Vollmer, 2008).

Table 1. Relative abundance(%)[b] of muropeptides from *K. setae*.

Muropeptide[a]	*K. setae*				
Monomers	16h	30h	42h	64h	spores
Mono	2.1	2.3	2.4	0.2	0.0
Di	24.5	27.7	27.4	28.8	22.6
Tri	6.0	9.1	6.8	6.2	8.2
Tri (-Gly)	9.8	10.9	13.0	10.4	5.3
Tetra	5.7	7.4	12.8	27.5	51.2
Tetra (-Gly)	37.2	34.3	30.9	21.3	7.3
Penta	0.4	0.2	0.4	1.2	2.5
Penta (-Gly)	13.9	7.9	6.1	3.5	0.5
Penta [Gly5]	0.4	0.2	0.2	0.9	2.3
Deacetylation	0.3	0.8	1.2	1.0	0.9
	K. setae				
Dimers	16h	30h	42h	64h	spores
TriTri [deAc/deAc]	13.7	11.9	15.5	5.3	1.3
TriTri (-GM/ -Gly)	4.0	6.0	3.9	1.6	2.1
TriTri (-GM) [deAc]	12.1	22.6	21.2	10.0	1.8
TriTetra	0.0	0.0	0.0	3.6	6.5
TriTetra [deAc]	23.0	11.3	11.6	4.6	0.3
TriTetra (-GM)	1.8	0.1	1.3	6.6	16.5
TriTetra (-GM) [deAc]	34.1	32.2	25.6	12.4	0.4
TriTetra (-GM) (-Gly)	3.7	6.5	4.7	2.8	1.8
TetraTetra	0.5	0.5	2.7	17.8	25.5
TetraTetra [deAc] [Gly4]	3.9	1.3	0.6	0.1	0.0
TetraTeta [Gly4]	2.6	6.8	7.1	18.1	7.7
TetraTetra (-GM)	0.5	0.8	5.8	17.0	38.1
Deacetylation	86.8	79.3	74.6	32.5	3.8
missing MurNAcGlcNAc	56.2	68.2	62.5	50.5	60.7
3-3 cross-links	92.4	90.7	83.8	47.1	28.8

[a] The labels correspond to the labels in Figure 2. (-Gly) misses the DAP-linked Gly, [Gly4] or [Gly5] has Gly instead of the 4th or 5th D-Ala, [deAc] carries MurN instead of MurNAc, (-G) lacks GlcNAc, (-GM) lacks GlcNAcMurNAc. Monomers and dimers are treated as separate datasets.

[b]Relative abundance is calculated as the ratio of the peak area over the sum of all peak areas recognized in the chromatogram.

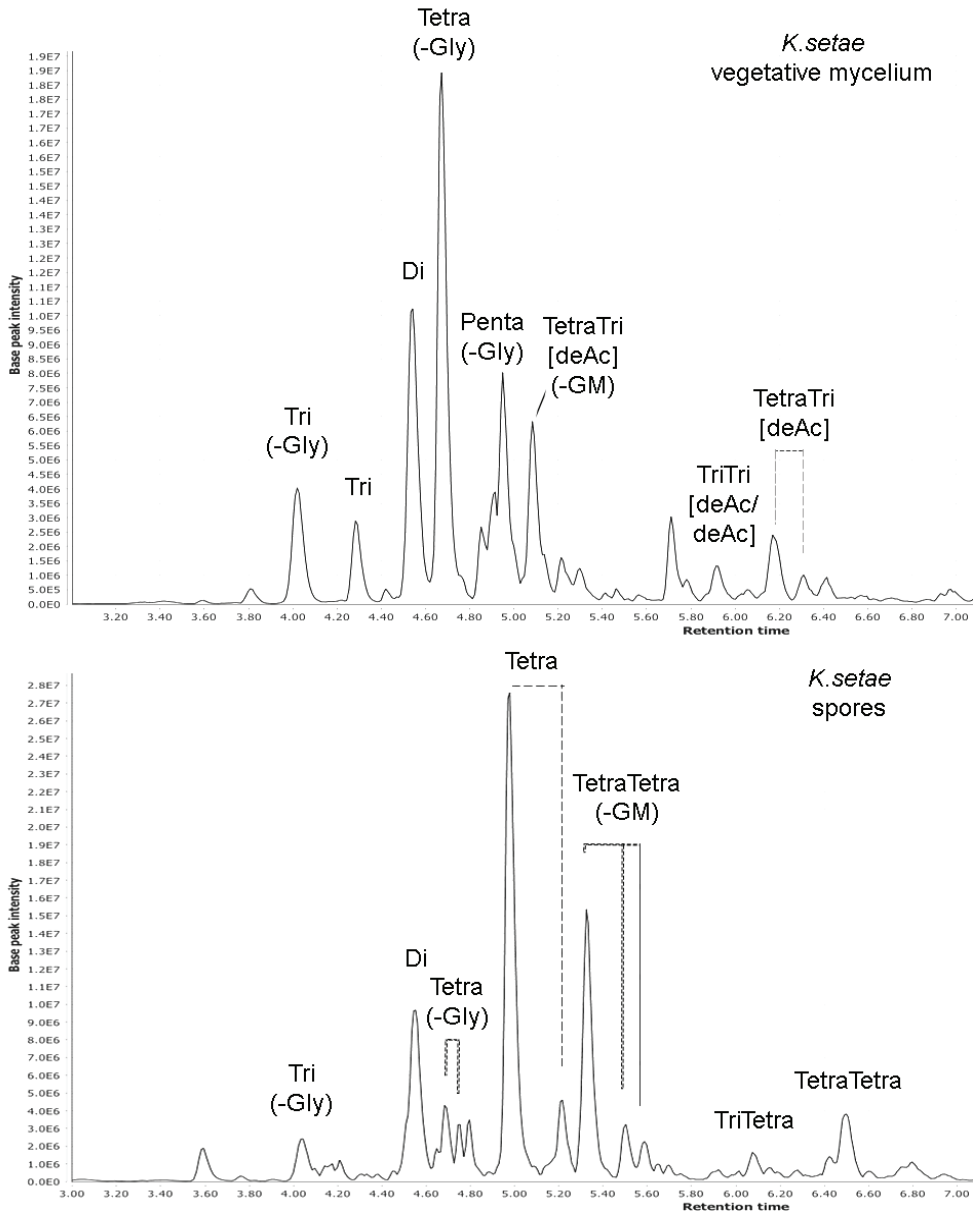

Figure 2. Separation of muropeptides from vegetative cells (top) and spores (bottom) of *K. setae*. Major peaks are labeled and correspond to the labels in Table 1. In vegetative PG, the major peaks are tetrapeptides lacking Gly, Tetra(-Gly), and pentapeptides lacking Gly, Penta(-Gly) with a high amount of de-acetylated, [deAc], dimers. In spore PG, the major peaks are tetrapeptides (Tetra) and TetraTetra dimers lacking GlcNac-MurNAc, TetraTetra(-GM). The x-axis shows the retention time in minutes, the y-axis the base peak intensity in arbitrary units.

Table 2. Relative abundance(%)[b] of muropeptides from *K. viridifaciens*

Muropeptides [a]	*K. viridifaciens*					
Monomers	16h	30h	42h	64h	spores	Δ*ssgB*
Mono	1.4	1.3	1.8	1.8	2.3	0.5
Di [deAc]	0.1	0.2	0.3	0.3	0.2	0.1
Di	8.4	11.6	14.6	13.7	7.5	8.5
Tri (-Gly)	1.0	0.9	1.1	0.9	0.7	0.5
Tri [NH2]	0.2	1.1	1.1	9.4	39.9	6.1
Tri-X	0.3	2.4	0.1	0.6	0.1	14.1
Tri (-G) [deAc]	0.4	0.6	0.7	1.5	2.4	0.6
Tri	31.6	27.3	31.7	24.2	17.3	16.9
Tetra (-Gly)	0.8	0.7	0.7	0.9	0.7	0.4
Tetra [Gly4]	2.2	0.9	0.8	0.8	0.5	0.4
Tetra-X	0.2	2.6	0.1	0.5	0.3	14.8
Tetra	29.5	31.3	28.9	31.5	22.8	17.8
Penta [Gly5]	3.1	1.6	1.6	1.3	1.0	0.7
Penta -X	0.2	1.4	0.1	0.3	0.1	8.9
Penta	20.6	16.2	16.9	12.7	4.2	9.6
Deacetylation	0.4	0.8	1.0	1.8	2.7	0.7
amidation DAP	0.2	1.1	1.1	9.4	39.9	6.1
Modification 'X'[c]	0.8	6.4	0.2	1.4	0.5	37.8
	K. viridifaciens					
Dimers	16h	30h	42h	64h	spores	Δ*ssgB*
TriTri	1.4	1.2	2.9	1.1	3.4	1.5
TriTri (-GM)	6.1	4.6	8.1	6.3	3.2	7.8
TriTetra	35.1	33.0	31.5	31.5	45.0	25.5
TriTetra (-GM) (-Gly)	2.8	3.2	3.3	2.3	0.7	2.5
TriTetra (-GM)	5.5	16.4	16.0	15.0	8.0	22.4
TetraTetra	19.9	18.1	18.3	20.4	13.7	12.9
TetraTetra [Gly]	1.2	3.0	0.5	1.3	12.9	2.3
TetraTetra (-GM)	16.6	11.9	7.4	10.5	7.3	15.7
TetraPenta	9.7	6.1	9.6	8.8	2.9	7.9
missing MurNAcGlcNAc	31.0	36.1	34.9	34.0	19.3	48.4
3-3 cross links	50.9	58.4	61.9	56.1	55.8	56.5

[a] The labels correspond to the labels in Figure 3. (-Gly) misses the DAP-linked Gly, [Gly4] or [Gly5] has Gly instead of the 4th or 5th D-Ala, [deAc] carries MurN instead of MurNAc, (-G) lacks GlcNAc, (-GM) lacks GlcNAcMurNAc.

[b]Relative abundance is calculated as the ratio of the peak area over the sum of all peak areas recognized in the chromatogram.

[c] The molecular mass assumed with '-X' is 2.01 Da.

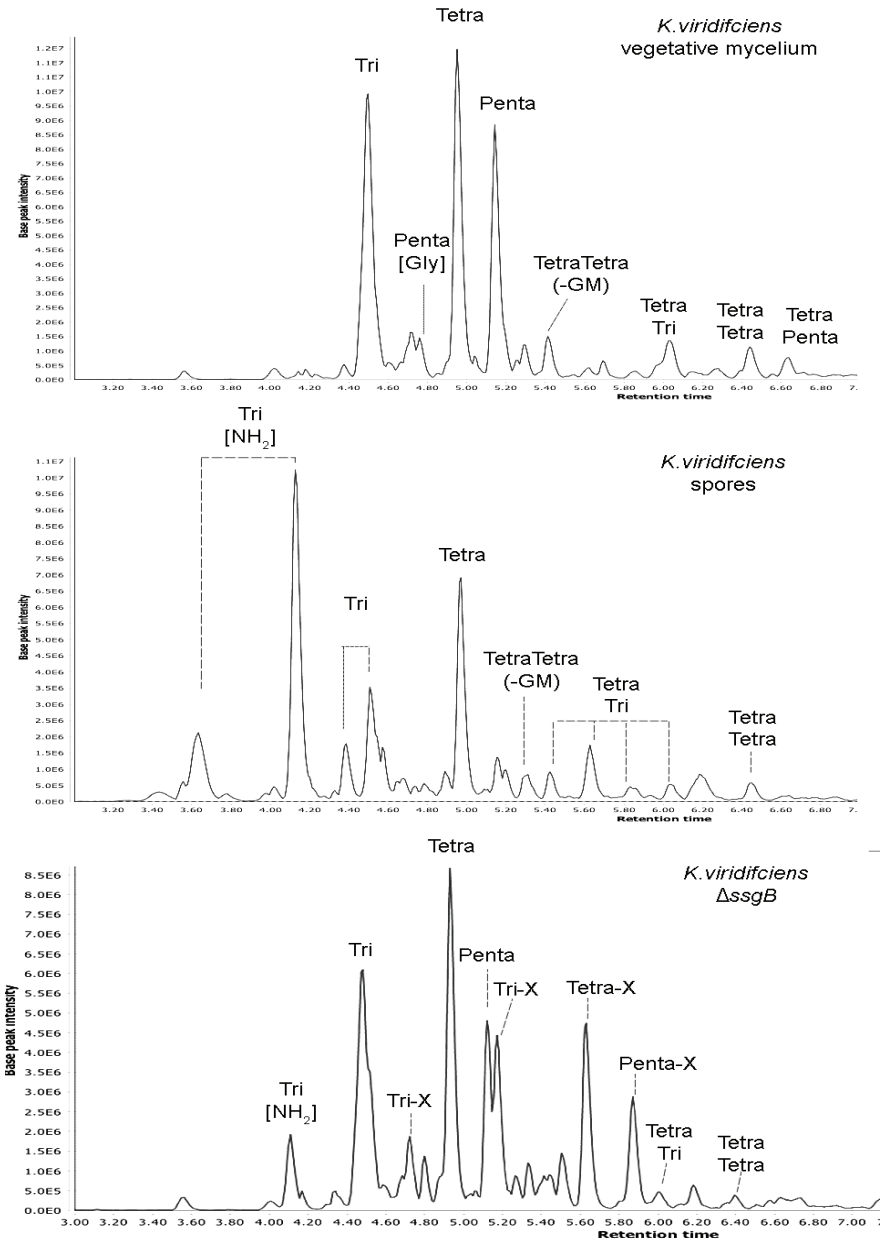

Figure 3. separation of muropeptides from *K. viridifaciens* vegetative mycelium (top), spores (middle) and the Δ*ssgB* deletion mutant (bottom). Major peaks are labeled and correspond to the labels in Table 2. Major peaks in vegetative mycelium are tripeptides, tetrapeptides and pentapeptides, Tri, Tetra and Penta. In spores, the major peaks are tripeptides with amidated DAP, Tri [NH_2] Tetra and the dimer consisting of a tetrapeptide and tripeptide, TetraTri. The muropeptide profile of the *ssgB* deletion mutant shows major amounts of muropeptides with modification 'X'. The x-axis shows the retention time in minutes, the y-axis the base peak intensity in arbitrary units.

In *K. setae*, the primary modifications in vegetative cells are the loss of the DAP-bound Gly and the de-acetylation of MurNAc, giving MurN, as shown in Figure 2 and Table 1. The major muropeptides seen in vegetative cells after 16 h of growth were tetrapeptides lacking Gly (Tetra (-Gly)) and dipeptides (Di), making up 37.2% and 24.5% of the total monomer content, respectively. This switches to tetrapeptides with Gly (tetra) and dipeptides (Di) at 51.2% and 22.6% of the monomers in spores. The amount of pentapeptides (-Gly) is higher in vegetative cells (14.5% of monomers) than in spores (3% of monomers).

The PG profile of *K. viridifaciens* is shown in Figure 3 and Table 2. The main difference between vegetative cells and spores was the amidation of DAP in spores. Vegetative hyphae of *K. viridifaciens* grown for 16 h had large amounts of tri-, tetra- and pentapeptides, 31.6%, 29.5% and 20.6% of the total content, respectively. In spores, this changed to tripeptides with an amidated DAP (Tri [NH_2]) and tetrapeptides (tetra), making up 39.9% and 22.8% of the total, respectively. The total monomer fraction with an amidated DAP was 40% in spores, whereas this modification was present only in trace amounts in vegetative mycelia (0.2 and 1.1% after 16 and 42 h, respectively). After 64 h, 9.4% of the muropeptides carried an amidated DAP, consistent with the formation of spores. Spore monomers carry a low amount of de-acetylated monomers, though a specific muropeptide contributed to this amount, MurN-tripeptides as have been found in *S. coelicolor,* mostly as LL-DAP (1.9% LL-DAP, 0.5% *meso*-DAP).

Modifications to dimers

Dimers are cross-linked via the DAP-bound Gly and, like in *Streptomyces*, *Kitasatospora* PG contains both 3-3 and 3-4 cross-links. Penicillin Binding Proteins (PBPs) recognize a pentapeptide as a donor strand, catalyze the cross-link of an DAP-bound Gly to D-Ala(4) on the opposite strand and cleave off the terminal D-Ala from the pentapeptide donor strand. L,D-transpeptidases recognize tetrapeptides and catalyze a cross-link between DAP-bound Gly and DAP, this way generating a 3-3 cross-link, and finally remove the terminal D-Ala from the tetrapeptide donor strand. TetraTetra dimers are cross-linked by a 3-4 cross-link, while TriTri dimers are cross-linked by a 3-3 cross-link. However, TetraTri dimers can be cross-linked by either 3-3 or 3-4 bonds; these two isomers have the same mass but they can be discriminated based on the difference in retention times. The cross-linking pattern was resolved by MS/MS (data not shown). In addition, cleavage of the glycan strand at the lactyl bond between MurNAc and L-Ala will give rise to dimers lacking one set of glycans.

Analysis of the PG dimers of *K. setae* showed that deacetylation of the glycan strands occurred mostly in vegetative cells (86.8% at 16 h) and far less frequently in spores (3.8%). In vegetative cells, both dimers with a double and a single set of glycans shows de-acetylated MurNAc, where 80% of the TetraTetra dimers lacking a set of glycans still caries MurN, suggesting that amidases are more likely to cleave off the dimers containing MurNAc than those containing MurN. Conversely, *K. setae* has an exceptionally high amount of dimers that have lost a set of glycans, representing 56-68% of the dimers throughout growth. The amount of 3-3 cross-links is higher in vegetative mycelium than in spores, at 92.4% at 16 h, 90.7 % at 30 h, 83.8 at 42 h, 47.1 at 64 h and 28.8% in spores. The PG dimers of *K. viridifaciens* had undergone less extensive hydrolysis as compared to those of *K. setae*, whereby 31-36% of the dimers of 16-64 h old cultures lacked a set of glycans, compared to

50-68% in *K. setae*. The amount of 3-3 cross-links in *K. viridifaciens* was relatively stable over time, between 50 and 62%.

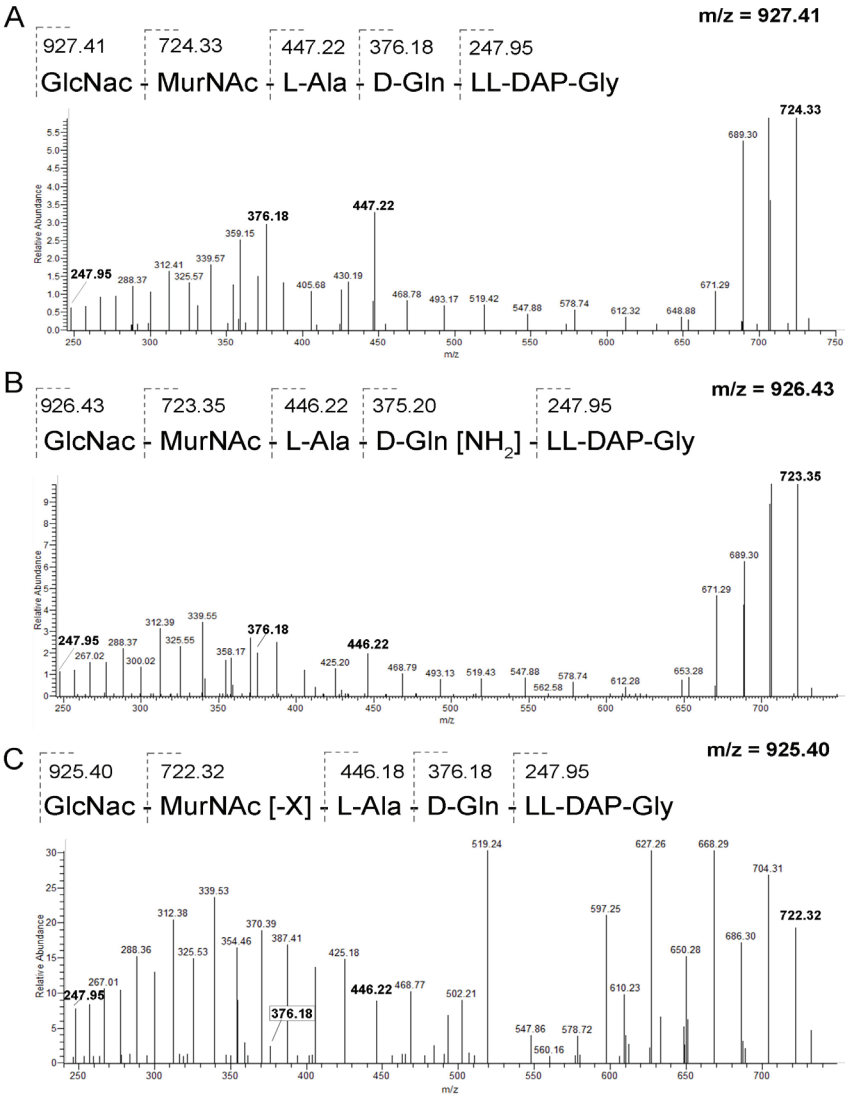

Figure 4. MS/MS fragmentations of (A), a tripeptide (Tri), which has a m/z of 927.41, (B) a tripeptide with an amidated DAP (Tri [NH_2]) , with an m/z of 926.43, and (C) a tripeptide with a 'X' modification (Tri [-X]) with an m/z of 925.40. The base structure shows the major fragment masses, which are given in bold in the mass spectrum.

Transition from mycelium to spores

The *ssgB* deletion mutant of *K. viridifaciens* forms normal vegetative and aerial mycelia but does not form spores. Studying this mutant therefore allows us to observe modifications specific for older aerial mycelia even after prolongued incubation, without having to take spores into account. Interestingly, the muropeptide profile of the *ssgB* null mutant contained many muropeptides with an unknown modification, here named 'X'. Canonical tripeptides have a mass of (M+H) 927.41, while the Tri [-X] peptides from *ssgB* mutants had a mass of (M+H) 925.40. Therefore, the 'X' modification represents a 2.01 Da mass reduction as compared to other muropeptides. This suggests that a double bond is present in the Tri [-X] while the bond is reduced in canonical tripeptides. After recognizing this modification in the *ssgB* mutant, we revisited the PG profiles of *K. viridifaciens* and scrutiny of these samples also identified minor amounts of modification 'X' in 30 h old samples, namely 5.7% of the monomers. Suggestively, this is the moment the strain has produced aerial hyphae but does not yet form spores. Modification 'X' most likely occurs at the MurNAc moiety, as MS/MS analysis showed the initial loss of GlcNAc, and this still showed the mass difference, while the mass difference was resolved at the pentapeptide chain (see Figure 4).

DISCUSSION

Kitasatosporae are characterized by the striking difference in stereochemistry of DAP between vegetative and spore PG (Takahashi, 2017). We show that besides the DAP stereochemistry, the entire PG composition changes between these two growth phases in their life cycle. Despite the similarity in their life cycles, this has not been seen in *Streptomyces* (van der Aart *et al.*, 2018). Vegetative mycelia of *K. setae* lack the DAP-bound Gly and has heavily de-acetylated MurNAc, whereas spore PG carries the DAP-bound Gly and shows less de-acetylation. *K. viridifaciens* vegetative mycelium carries no modifications, whereas spores carry *N*-deacetylated MurN lacking GlcNAc, called MurN-Tri, and a large amount of amidated DAP. The amidation of DAP in *K. viridifaciens* occurs mostly in the spore wall, where 40% of all monomers carry an amidated version of DAP, in contrast to 0.2% in the 16 h old vegetative mycelium. Interestingly, 6.1% of the DAP residues in the cell wall of the non-sporulating *ssgB* mutant was amidated. Amidation of DAP contributes to lysozyme resistance by slightly decreasing the negative charge of the PG, that way reducing affinity of the positively charged lysozyme (Levefaudes *et al.*, 2015). However, this modification was not found in *K. setae*, and is therefore not a trait that is typical of all members of the genus *kitasatospora*.

PG cross-linking is performed by two different types of proteins, penicillin binding proteins (PBPs) and L, D-transpeptidases (LDTs). PBPs produce canonical 3-4 cross-links between the DAP-bound Gly and the 4th amino acid of the next strand, D-Ala. LDTs produce 3-3 cross-links between the DAP-bound Gly and the 3rd amino acid of the next strand, DAP. Tip growing bacteria have a relatively high amount of 3-3 cross-links due to the remodeling of the lateral wall by LDTs (Baranowski *et al.*, 2018). We have shown that in *Kitasatospora* the cross-linking is similar as in *Streptomyces*, as was apparent from the MS/MS patterns. In *K. setae*, the amount of 3-3 cross-links is high in vegetative (92% at 16 h) and lower during sporulation (47% at 64 h) and in spore preparations (29%). In *K. setae* and in *S. coelicolor* the proportion of 3-3 cross-links is higher in vegetative mycelium than in spores, which makes a correlation to specific DAP-isomers unlikely. In *K. viridifaciens*, the amount of 3-3 cross-links remained stable throughout growth at 50-62%, in contrast to what was observed for the PG of *K. setae*, where the amount of 3-3 cross-links was growth phase-dependent. The high amount of dimers lacking a set of glycans in *K. setae* is striking, as this type of dimer does not contribute to strengthening the PG structure (Vollmer *et al.*, 2008). Even of the dimers found in spores 61% lacked glycans, whereby a TetraTetra dimer lacking GlcNAc-MurNAc (TetraTetra(-GM)) prevailed (38.1%). In *K. viridifaciens*, the amount of dimers lacking glycans was lower, namely 31%- 36% in vegetative mycelium and 19.3% in spores.

The cell wall of the *ssgB* null mutant of *K. viridifaciens* showed a mass that could not be explained, and may therefore represent a unique modification. Ms/Ms anbalysis showed that the loss of 2.01 Da took place at the MurNac moiety, and could be accounted for by formaitn of a double bond and hence loss of two hydrogens, such as in non-reduced MurNAc. The modification is highly abundant in the peptidoglycan of the *ssgB* mutant, but also occurred in small amounts over the course of growth, especially after 30 h of growth, coinciding with the onset of aerial mycelium formation by *K. viridifaciens*. This is precisely the stage where *ssgB* mutants are blocked in development, and we may therefore see the consequence of a specific cell-cycle arrest. Modification “X” may be part of a hydrolytic event. Peptidoglycan amidases

recognize tri-, tetra- and pentapeptides and targets the site between MurNac and L-Ala, then the muropeptide is cleaved between the MurNAc-associated D-Lac and L-Ala (Rocaboy *et al.*, 2013; Wang *et al.*, 2003). The exact nature of the '-X' modification requires further investigation.

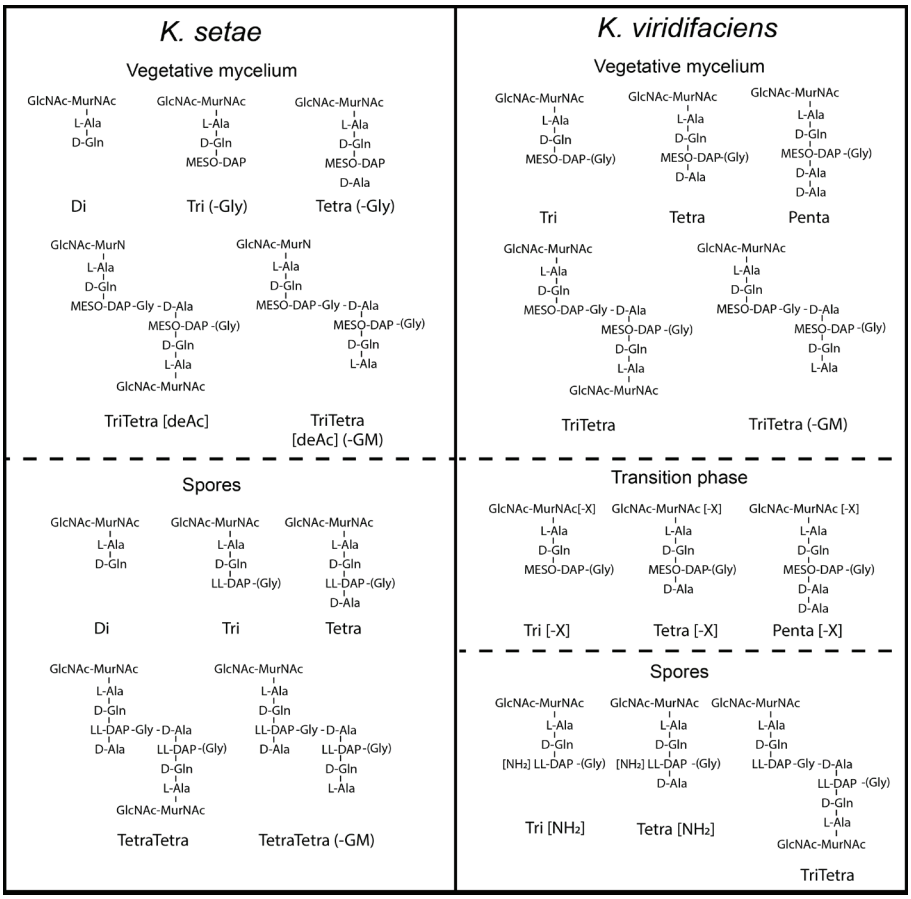

Figure 5. summary of muropeptides found in *K. setae* and *K. viridifaciens* vegetative mycelium and spores. Vegetative mycelium of *K. setae* shows muropeptides with Meso-DAP, lacking Gly (-Gly) and with de-Acetylated MurNAc [deAc], spore cell walls carry muropeptides with LL-DAP, Gly and less deacetylation. *K. viridifaciens* carries Meso-DAP in vegetative mycelium, in transition phase, modification X occurs at the site of MurNAc. Spore mycelium shows LL-DAP and amidated DAP [NH_2]. In all life stages, some dimers lack a set of glycans (-GM).

Cell wall construction

DapF is responsible for the isomerization of LL-DAP into *meso*-DAP, while *K. setae* has 3 copies of this gene, other species of *Kitasatospora* have a single copy (Girard *et al.*, 2014). *Streptomyces* species, which only have LL-DAP, also carry DapF but *meso*-DAP can be an intermediate in the production of Lysine. During the process of cell wall construction, the well-conserved MurE adds LL- or *meso*-DAP to

UDP-MurNac-L-Ala-D-Glu (Smith, 2006). The only other bacterium that carries both LL- and *meso*-DAP, the Gram-negative *Myxococcus xanthus*, has a single version of MurE (Bui *et al*., 2009). *Kitasatosporae* apparently carry two versions of MurE, one of which is encoded by a gene in the *dcw* (division of cell wall) cluster and one by a gene elsewhere on the genome. It needs to be analyzed whether the second MurE paralogue is functional and if so, whether it has similar bioactivity as the canonical MurE. In any case, the *murE* genes of *Kitasatosporae* (LL- and *meso*-DAP) are placed in a separate clade from *Streptomyces* (LL-DAP) and *Mycobacterium* (*meso*-DAP), suggesting that the canonical MurE could be adapted to ligate both LL- and *meso*-DAP (Hwang *et al*., 2015).

Implications for taxonomy

The switch from incorporation of *meso*-DAP to incorporating LL-DAP in the PG allows us to recognize the difference between these different types of mycelia, which is more elusive in *Streptomyces*. Notably, *Kitasatosporae* appear to hydrolyze most of the mycelia with *meso*-DAP at the switch to spore formation, and although the concept of programmed cell death is not novel, the hydrolysis of practically all PG with *meso*-DAP comes as a surprise. After 64 h, the cell wall of both *Kitasatosporae* mostly contains LL-DAP, indicating that extensive hydrolysis of the vegetative mycelium has taken place. This implies that if chemotaxonomy is performed on older cultures of *Kitasatosporae*, the data could be confused with those obtained for *Streptomyces*. Previous amino acid composition analyses had already shown that *K. setae* carries very little glycine in the vegetative mycelium, but an equal amount of Gly per DAP in spores (Takahashi *et al*., 1999). However, this shift in the amount of Gly is not specific for *Kitasatosporae*, as *K. viridifaciens* has equal amounts of Gly per DAP throughout its life cycle.

In summary, our data show that *Kitasatosporae* have different modifications in vegetative mycelium and in spores, besides just the switch from *meso*-DAP to LL-DAP. This switch requires different modifying and hydrolyzing proteins and emphasizes that tip growth and sporulation are regulated by two different systems. Mycelium of the *ssgB* contained a yet unidentified modification, which suggests loss of 2.01 Da in the MurNAc moiety. The presence of only trace amounts of LL-DAP in this mutant indicates that the DAP stereochemistry of the *Kitasatospora* cell wall changes at a time prior to the onset of septation. It is difficult to say whether the difference in stereochemistry is a consequence or a cause of the switch in regulation between vegetative- and reproductive growth.

Acknowledgements

The *ssgB* mutant of *K. viridifaciens* was a kind gift from Karina Ramijan and Dennis Claessen.

Appendix 1. Supplemental information belonging to Chapter 4

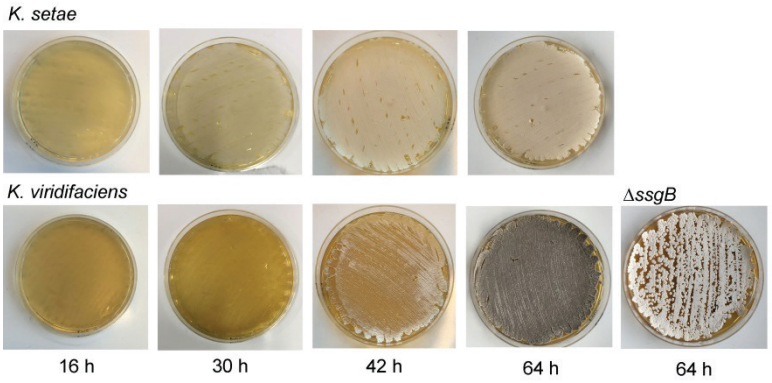

Figure S1. Development of *K. setae* and *K. viridifaciens* grown on SFM agar plates. After 42 h, *K. setae* had developed white spores, as seen with an impression print, while *K. viridifaciens* developed white aerial mycelium at 42 h and grey spores after 64 h. The *ssgB* mutant of *K. viridifaciens* only formed aerial hyphae and did not develop spores.

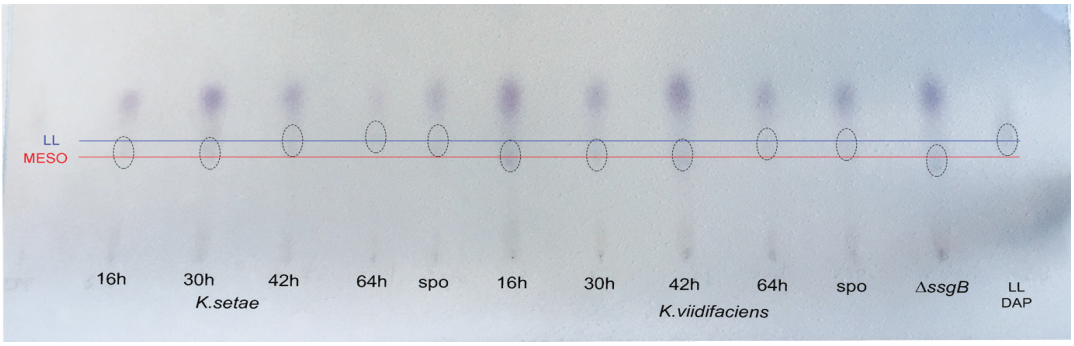

Figure S2. DAP analysis of *K.setae* and *K.viridifaciens* over growth. At 16 h, both strains only carry *meso*-DAP in the PG, after 42 hours this shifts to LL-DAP. At 64 h both strains carry mostly LL-DAP. The *ssgB*-mutant consists mostly of *meso*-DAP but has trace amounts of LL-DAP.

Table S1. Muropeptides identified in *K. setae* peptidoglycan

Monomers	Observed mass		*K. setae*				
	[M+H]	rt	16 h	30 h	42 h	64 h	spores
Mono	570.25	4.68	2.1%	2.3%	2.4%	0.2%	0.0%
Di [deAc]	656.30	4.08	0.3%	0.8%	1.2%	1.0%	0.9%
Di	698.31	4.54	20.2%	22.5%	21.6%	22.9%	17.5%
Di [Glu]	699.30	4.99	4.0%	4.4%	4.6%	4.9%	4.2%
Tri (-Gly)	870.40	4.02	8.5%	9.5%	10.9%	8.9%	4.7%
Tri (-Gly) [Glu]	871.38	4.29	1.3%	1.4%	2.1%	1.5%	0.6%
Tri	927.42	4.28	5.1%	7.6%	4.2%	2.5%	0.0%
Tri	927.42	4.52	0.0%	0.3%	1.5%	3.0%	6.2%
Tri [Glu]	928.40	4.76	0.0%	0.0%	0.1%	0.3%	0.7%
Tri [Glu]	928.40	4.51	0.9%	1.2%	0.7%	0.0%	0.0%
Tetra (-Gly)	941.43	4.67	32.8%	28.4%	25.1%	17.8%	6.4%
Tetra (-Gly) [Glu]	942.42	4.92	4.4%	5.9%	5.8%	3.6%	0.9%
Tetra	998.46	4.53	3.5%	3.2%	2.0%	0.0%	0.0%
Tetra	998.46	4.97	0.7%	3.2%	8.8%	23.4%	44.0%
Tetra [Glu]	999.44	4.76	0.7%	0.4%	0.0%	0.0%	0.0%
Tetra [Glu]	999.44	5.19	0.7%	0.6%	2.1%	4.1%	7.2%
Penta [deAc]	1012.47	4.95	13.8%	6.7%	5.2%	3.0%	0.4%
Penta [Glu/ deAc]	1013.46	5.21	0.1%	1.1%	0.9%	0.5%	0.1%
Penta [Gly5]	1055.48	4.78	0.1%	0.1%	0.1%	0.9%	2.2%
Penta [Gly5]	1055.48	4.29	0.0%	0.1%	0.1%	0.0%	0.0%
Penta [Glu /Gly5]	1056.46	4.61	0.3%	0.0%	0.0%	0.0%	0.0%
Penta	1069.49	5.18	0.0%	0.2%	0.4%	1.1%	2.2%
Penta [Glu]	1070.48	5.44	0.3%	0.0%	0.1%	0.1%	0.3%
Dimers							
	[M+H]	rt	16 h	30 h	42 h	64 h	spores
TriTri (-GM /-Gly)	1298.60	4.59	2.1%	4.0%	1.7%	1.1%	1.8%
TriTri (-GM /-Gly) [Glu]	1299.60	4.60	1.9%	2.0%	2.2%	0.6%	0.3%
GnacMN-tritri	1313.60	5.06	1.8%	4.2%	3.7%	1.9%	0.6%
GnacMN-tritri	1313.60	5.61	3.7%	8.9%	7.8%	3.1%	0.5%
GnacMN-tritri	1314.60	5.60	1.8%	0.7%	3.3%	2.2%	0.4%
GnacMN-tritri	1314.62	4.91	4.8%	8.9%	6.4%	2.8%	0.4%
TetraTri (-GM) (-Gly)	1369.64	4.63	1.9%	5.8%	2.9%	1.9%	0.9%

TetraTri (-GM) (-Gly)	1370.63	4.80	1.8%	0.7%	1.8%	0.9%	0.8%
GMN-TetraTri	1383.65	5.08	27.3%	26.0%	20.0%	9.8%	0.4%
GMN-TetraTri	1384.64	5.27	6.8%	6.2%	5.6%	2.6%	0.0%
TetraTri (-GM)	1426.66	5.01	0.0%	0.1%	0.5%	4.9%	12.0%
TetraTri (-GM) [Glu]	1427.66	4.95	1.8%	0.0%	0.7%	1.7%	2.5%
TetraTri (-GM) [Glu,-Glu]	1497.68	4.97	0.0%	0.0%	0.0%	0.0%	2.0%
GM-tetratetra	1497.70	5.32	0.3%	0.6%	3.2%	15.5%	30.8%
GM-tetratetra	1498.68	5.48	0.2%	0.2%	2.6%	1.5%	5.2%
TriTri [deAc,deAc] (3-3)	1792.81	5.94	8.6%	8.3%	7.0%	2.3%	0.7%
TriTri [deAc,deAc] (3-3)	1793.80	6.13	2.5%	0.5%	5.3%	2.1%	0.4%
TriTri [deAc,deAc] (3-3)	1794.82	5.92	2.5%	3.0%	3.2%	0.9%	0.3%
TetraTri [deAc] (3-3)	1863.85	6.18	22.2%	9.5%	5.9%	1.8%	0.1%
GnacMN-tritetraGM (3-3)	1864.84	6.41	0.8%	1.8%	5.7%	2.8%	0.1%
GnacMnac-AEm(G) A-(G)mEA-GM	1906.86	6.08	0.0%	0.0%	0.0%	1.8%	3.9%
GnacMnac-AEm(G) A-(G)mEA-GM	1906.86	6.02	0.0%	0.0%	0.0%	0.5%	0.7%
GM-tri-tetra-MG	1907.84	6.25	0.0%	0.0%	0.0%	0.1%	0.2%
GM-tri-tetra-MG	1907.86	6.03	0.0%	0.0%	0.0%	1.2%	1.7%
TetraTetra [Gly4 / deAc]	1934.89	6.31	3.9%	1.3%	0.6%	0.1%	0.0%
TetraTetra [Gly4 / Glu]	1964.76	4.54	0.8%	2.1%	4.4%	3.7%	2.2%
TetraTetra [Gly4 / Glu]	1964.76	4.96	1.3%	2.3%	0.8%	5.5%	4.9%
TetraTetra [Gly4 / Glu]	1964.76	6.08	0.3%	1.2%	0.8%	3.7%	0.3%
TetraTetra [Gly4 / Glu]	1964.78	6.41	0.3%	1.2%	1.2%	5.2%	0.3%
GM-tetra-tetra-MG	1977.90	6.50	0.2%	0.3%	1.2%	5.4%	11.1%
GM-tetra-tetra-MG	1978.88	6.76	0.2%	0.1%	0.7%	6.1%	2.7%
GM-tetra-tetra-MG	1978.90	6.49	0.2%	0.1%	0.7%	6.3%	11.4%
GM-tetra-tetra-MG	1979.39	6.50	0.0%	0.0%	0.0%	0.0%	0.3%

Table S2. Muropeptides identified in *K. viridifaciens* peptidoglycan

	Monomers	observed mass		*K. viridifaciens*					
m/z	ID	[M+H]	rt (min)	16 h	30 h	42 h	64 h	spores	Δ*ssgB*
570.25	Mono	570.25	4.67	1.4%	1.3%	1.8%	1.8%	2.3%	0.5%
656.30	Di [deAc]	656.30	4.07	0.1%	0.2%	0.3%	0.3%	0.2%	0.1%
682.27	Tri [deAc] (-G)	682.27	4.99	0.0%	0.0%	0.1%	1.0%	1.9%	0.0%
682.30	Tri [deAc] (-G)	682.30	4.58	0.4%	0.6%	0.6%	0.5%	0.5%	0.6%
698.31	Di	698.31	4.52	7.0%	10.1%	12.3%	11.5%	6.3%	7.3%
699.30	Di [Glu]	699.30	4.98	1.4%	1.3%	2.0%	1.9%	0.9%	1.1%
724.34	Tri (-G)	724.34	4.49	2.1%	1.9%	2.1%	1.6%	0.7%	1.1%
725.32	Tri (-G) [Glu]	725.32	4.71	0.3%	0.1%	0.2%	0.2%	0.0%	0.1%
781.36	Tetra (-G) [Gly4]	781.36	4.55	0.1%	0.1%	0.0%	0.0%	0.0%	0.0%
795.38	Tetra (-G)	795.38	4.95	1.7%	2.0%	1.8%	2.1%	1.4%	1.2%
796.36	Tetra (-G) [Glu]	796.36	5.18	0.2%	0.1%	0.2%	0.2%	0.1%	0.1%
870.40	Tri (-Gly)	870.40	4.02	0.9%	0.9%	1.0%	0.9%	0.7%	0.5%
871.38	Tri (-Gly) [Glu]	871.38	4.28	0.1%	0.1%	0.1%	0.1%	0.1%	0.0%
925.40	Tri-X	925.40	4.72	0.1%	0.7%	0.0%	0.2%	0.1%	3.8%
925.40	Tri-X	925.40	5.17	0.2%	1.7%	0.0%	0.4%	0.1%	10.3%
926.43	Tri [NH2]	926.43	3.63	0.0%	0.0%	0.0%	0.4%	9.9%	2.7%
926.43	Tri [NH2]	926.43	4.12	0.2%	1.1%	1.1%	9.0%	30.1%	3.4%
927.42	Tri	927.42	4.39	0.0%	0.0%	0.0%	0.0%	5.0%	0.0%
927.42	Tri	927.42	4.50	25.0%	22.8%	26.2%	19.9%	10.2%	13.9%
928.40	Tri [Glu]	928.40	4.71	4.2%	2.4%	3.2%	2.5%	1.4%	1.8%
941.43	Tetra (-Gly)	941.43	4.67	0.8%	0.7%	0.7%	0.9%	0.7%	0.4%
984.44	Tetra [Gly4]	984.44	4.55	1.9%	0.9%	0.7%	0.7%	0.5%	0.4%
985.42	Tetra [Glu/Gly4]	985.42	4.75	0.2%	0.0%	0.0%	0.0%	0.1%	0.0%
996.44	Tetra -X	996.44	5.22	0.0%	0.6%	0.0%	0.0%	0.0%	3.8%
996.44	Tetra -X	996.44	5.69	0.2%	1.4%	0.0%	0.4%	0.2%	8.3%
997.43	Tetra [Glu/ -X]	997.43	5.89	0.0%	0.3%	0.0%	0.1%	0.0%	2.0%
997.43	Tetra [Glu / -X]	997.43	5.39	0.0%	0.3%	0.0%	0.0%	0.0%	0.8%
998.46	Tetra	998.46	4.95	23.4%	26.0%	24.2%	25.9%	18.6%	14.6%
999.44	Tetra [Glu]	999.44	5.18	4.1%	3.1%	2.7%	3.4%	2.7%	2.0%
1055.48	Penta [Gly5]	1055.48	4.76	2.7%	1.5%	1.4%	1.2%	1.0%	0.6%
1056.46	Penta [Glu /Gly5]	1056.46	4.98	0.4%	0.1%	0.1%	0.1%	0.0%	0.0%
1067.48	Penta -X	1067.48	5.87	0.1%	0.4%	0.0%	0.2%	0.0%	5.2%
1067.48	Penta -X	1067.48	5.33	0.1%	0.9%	0.0%	0.0%	0.0%	2.0%

1068.46	Penta [Glu / -X]	1068.46	6.18	0.0%	0.1%	0.0%	0.0%	0.0%	1.2%
1068.46	Penta [Glu / -X]	1068.46	5.62	0.0%	0.0%	0.0%	0.0%	0.0%	0.5%
1069.49	Penta	1069.49	5.14	17.5%	14.5%	14.9%	11.2%	3.7%	8.4%
1070.48	Penta [Glu]	1070.48	5.41	3.2%	1.8%	2.0%	1.5%	0.5%	1.3%
Dimers	Dimers								
m/z	ID	[M+H]	rt(min)	16 h	30 h	42 h	64 h	spores	Δ*ssgB*
1298.60	TriTri (-GM /-Gly)	1298.60	5.07	2.9%	1.2%	2.9%	2.1%	2.0%	0.9%
1355.62	TriTri (-GM)	1355.62	4.60	3.2%	3.4%	5.2%	4.2%	1.2%	6.9%
1369.64	TetraTri (-GM) (-Gly)	1369.64	5.25	2.8%	3.2%	3.3%	2.3%	0.7%	2.5%
1426.66	TetraTri (-GM)	1426.66	4.93	5.5%	16.4%	16.0%	15.0%	8.0%	22.4%
1497.70	TetraTetra (-GM)	1497.70	5.30	5.0%	5.2%	4.4%	6.8%	3.9%	4.7%
1498.68	TetraTetra (-GM) [Glu]	1498.68	6.18	7.8%	4.5%	2.5%	3.1%	2.5%	8.5%
1499.68	TetraTetra (-GM) [Glu/Glu]	1499.68	6.16	3.7%	2.2%	0.6%	0.6%	0.9%	2.4%
1835.82	TriTri (3-3)	1835.82	5.62	1.4%	1.2%	2.9%	1.1%	3.4%	1.5%
1904.84	TetraTri [-X]	1904.84	6.62	0.0%	0.0%	0.0%	0.0%	0.0%	3.2%
1906.87	TetraTri (3-4)	1906.87	5.42	0.0%	0.0%	0.0%	0.0%	4.5%	0.0%
1906.87	TetraTri (3-3)	1906.87	5.63	0.7%	0.9%	0.4%	2.9%	20.1%	0.0%
1906.86	TetraTri (3-3)	1906.86	5.83	0.0%	0.0%	0.0%	0.0%	3.4%	0.0%
1906.86	TetraTri (3-3)	1906.86	6.04	17.4%	17.0%	16.3%	14.6%	9.4%	11.7%
1907.86	TetraTri [Glu] (3-3)	1907.86	6.03	17.0%	15.0%	14.8%	14.0%	7.6%	10.6%
1907.84	TetraTri [Glu] (3-4)	1907.84	6.25	1.9%	2.5%	2.3%	3.0%	2.8%	1.5%
1964.75	TetraTetra[Gly4]	1964.75	5.15	1.2%	3.0%	0.5%	1.3%	12.9%	2.3%
1977.89	TetraTetra	1977.89	6.45	8.6%	9.2%	9.1%	10.2%	8.8%	6.6%
1978.90	TetraTetra [Glu]	1978.90	6.37	11.2%	9.0%	9.2%	10.2%	4.9%	6.3%
2048.93	PentaTetra	2048.93	6.63	5.3%	2.3%	4.3%	3.4%	1.7%	3.8%
2049.93	PentaTetra [Glu]	2049.93	6.57	4.3%	3.8%	5.2%	5.4%	1.2%	4.1%

Supplementary table 3

The amino acid composition in the cell wall of *K.setae* was previously assessed to be:

Cell type	DAP	Ala	Glu	Gly
Aerial spores	1.0 (*LL*)	1.6	0.5	1.0
Vegetative mycelium	1.0 (*meso*)	1.6	0.7	0.2

Amino acid composition in the cell walls of K.setae (Molar ratio). Table adapted from (Takahashi *et al.*, 1999)